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**SKIN CARE PRODUCT CONTAINING RETINOIDS, RETINOID BOOSTER AND  
PHYTOESTROGENS IN A DUAL COMPARTMENT PACKAGE**

This application claims priority under 35 U.S.C. § 119 from provisional  
5 application Serial No. 60/258,457, filed December 28, 2000.

**FIELD OF THE INVENTION**

The invention relates to stable skin care compositions containing a retinoid in  
10 a first compartment and a retinoid booster system and a phytoestrogen in a second  
compartment of a dual compartment package.

**BACKGROUND OF THE INVENTION**

15 Retinoids (e.g. retinol and retinyl esters) are common ingredients used in  
cosmetic products. Retinol (vitamin A) is an endogenous compound which occurs  
naturally in the human body and is essential for normal epithelial cell differentiation.  
Natural and synthetic vitamin A derivatives have been used extensively in the  
treatment of a variety of skin disorders and have been used as skin repair or renewal  
20 agents. Retinoic acid has been employed to treat a variety of skin conditions, e.g.,  
acne, wrinkles, psoriasis, age spots and discoloration. See e.g. Vahlquist, A. et al.,  
"Isotretinoin Treatment of Severe Acne Affects the Endogenous Concentration of  
Vitamin A in Sebaceous Glands," J. Invest. Dermatol., Vol. 94, pp. 496-498 (1990),  
Ellis, C.N. et. Al., "Pharmacology of Retinols in Skin," Basel, Karger, Vol. 3, (1989),  
25 pp. 249-252; and PCT Patent Application No. WO 93/19743.

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Retinoid metabolism, however may result in conversion of the retinoid to non-beneficial by-products, thus yielding a lesser amount of beneficial retinoic acid to treat skin conditions. Several prior art references, therefore, teach the use of a variety of natural actives for aiding in the treatment of skin conditions such as acne, wrinkles, psoriasis, age spots, and discoloration. For example, phytoestrogens (i.e., natural compounds which have estrogen-like activity and which are found in plants) have been increasingly used for cosmetic and therapeutic purposes. Estrogens and synthetic compounds which act like estrogens are known to increase the thickness of the dermal layer and reduce the wrinkle formation in the aging skin. Changes in the skin such as skin dryness, loss of skin elasticity and plumpness occurring after menopause are attributed to the lack of estrogen production. Estrogen therapy prevents or slows down many of the changes associated with aging skin (4) (Creidi et al., "Effect of a Conjugated Oestrogen Cream (Premarin®) on Aging Facial Skin," Maturitas, 19, p. 211-213, 1994).

Several phytoestrogens have been disclosed in the prior art for cosmetic benefits. For example, U.S. Patent No. 5,728,726 teaches the use of genistein for tyrosine kinase inhibitory activity. U.S. Patent Nos. 5,847,003 and 5,834,513 assigned to Avon disclose the use of oxacids and oxadiacids in combination with retinoids. Both Avon patents disclose the use of antioxidant bioflavonoids, such as genistein and daidzein, as optional ingredients.

It has been discovered, however, that phytoestrogens induce oxidation of retinol, and therefore contribute to retinol degradation. Although multi-compartment systems for delivering compositions have been described in the prior art, none disclose the need to separate phytoestrogens from retinoids. For example, U.S. Patent No. 5,914,116 issued to the assignee of the present invention describes two

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separate containers for separating two different skin actives to provide dual skin benefits with one compartment containing retinoids and the second compartment containing a second active providing a second and different benefit.

5           Therefore, there still exists a need for compositions that provide the skin benefits of retinoids along with the retinoid enhancing benefits of phytoestrogens.

**SUMMARY OF THE INVENTION**

10           A stable skin care product containing:

          a first composition comprising about 0.001% to about 10% of a retinoid;

15           a second composition comprising about 0.0001% to about 50% of at least one retinoid booster and about 0.001% to about 10% of a phytoestrogen;

          a first compartment for storing the first composition; and

20           a second compartment for storing the second composition, the first and second compartments being joined together.

**DETAILED DESCRIPTION**

25           As used herein, the term "comprising" means including, made up of, composed of, consisting and/or consisting essentially of. Except in the operating and comparative examples, or where otherwise explicitly indicated, all numbers in

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this description indicating amounts or ratios of materials or conditions of reaction, physical properties of materials and/or use are to be understood as modified by the word "about".

5           The inventive compositions contain, as a preferred ingredient, a retinoid, which is selected from retinyl esters, retinol, retinal and retinoic acid, preferably retinol or retinyl ester. The term "retinol" includes the following isomers of retinol: all-trans-retinol, 13-cis-retinol, 11-cis-retinol, 9-cis-retinol, 3,4-didehydro-retinol, 3,4-didehydro-13-cis-retinol; 3,4-didehydro-11-cis-retinol; 3,4-didehydro-9-cis-retinol. Preferred  
10 isomers are all-trans-retinol, 13-cis-retinol, 3,4-didehydro-retinol, 9-cis-retinol. Most preferred is all-trans-retinol, due to its wide commercial availability.

Retinyl ester is an ester of retinol. The term "retinol" has been defined above. Retinyl esters suitable for use in the present invention are C<sub>1</sub>-C<sub>30</sub> esters of retinol, preferably C<sub>2</sub>-C<sub>20</sub> esters, and most preferably C<sub>2</sub>, C<sub>3</sub>, and C<sub>16</sub> esters because they  
15 are more commonly available. Examples of retinyl esters include but are not limited to: retinyl palmitate, retinyl formate, retinyl acetate, retinyl propionate, retinyl butyrate, retinyl valerate, retinyl isovalerate, retinyl hexanoate, retinyl heptanoate, retinyl octanoate, retinyl nonanoate, retinyl decanoate, retinyl undecanoate, retinyl laurate, retinyl tridecanoate, retinyl myristate, retinyl pentadecanoate, retinyl heptadecanoate,  
20 retinyl stearate, retinyl isostearate, retinyl nonadecanoate, retinyl arachidonate, retinyl behenate, retinyl linoleate, retinyl oleate.

The preferred ester for use in the present invention is selected from retinyl  
25 palmitate, retinyl acetate and retinyl propionate, because these are the most commercially available and therefore the cheapest. Retinyl linoleate and retinyl oleate are also preferred due to their efficacy.

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The diagram illustrates the retinoid cycle, showing the conversion of Retinyl Esters to Retinyl Ester, then to Retinol, Retinal, Retinoic Acid, and finally Degraded Products. Key enzymes involved include ARAT/LRAT (B1), Retinyl ester hydrolase, Retinol reductase (B3), Retinol dehydrogenase (B2), Retinal dehydrogenase, CRABP-2 (B4), and Cytochrome P450 (B5).

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graph LR
    RE[Retinyl Esters] --> RetE[Retinyl Ester]
    RetE -- "ARAT/LRAT (B1)" --> Ret[Retinol]
    Ret -- "Retinyl ester hydrolase" --> RetE
    Ret --> Retol[Retinol]
    RBP[RBP- Retinol (systemic)] --> Retol
    Retol -- "Retinol reductase (B3)" --> Retal[Retinal]
    Retal -- "Retinol dehydrogenase (B2)" --> Retol
    Retal -- "Retinal dehydrogenase" --> RetA[Retinoic Acid]
    RetA -- "CRABP-2 (B4)" --> DP[Degraded Products]
    RetA -- "Cytochrome P450 (B5)" --> DP
  
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15 CRABP II = Cellular Retinoic Acid Binding Protein II

It has been discovered, surprisingly, that certain compounds inhibit ARAT/LRAT, retinal reductase, CRABP II and retinoic acid oxidation (the latter catalyzed by cytochrome P450 systems), whereas certain other compounds enhance retinol dehydrogenase. The compounds are collectively termed herein as "boosters" and are coded as groups B1 through B5, as can be seen in Chart 1 hereinabove. The boosters, alone or in combination with each other, potentiate the action of a retinoid by increasing the amount of retinol available for conversion to retinoic acid and inhibiting the degradation of retinoic acid. The boosters act in conjunction with a retinoid (e.g. retinol, retinyl ester, retinal, retinoic acid), the latter being present endogenously in the skin. The preferred compositions, however, include a retinoid in the composition, co-present with a booster, to optimize performance.

The present invention includes, in part, a second composition containing about 0.0001% to about 50%, preferably about 0.001% to about 10%, most preferably about 0.001% to about 5% by weight of the composition of at least one booster compound, wherein the compound, either alone or at a combined concentration of 10mM, inhibits transglutaminase [in an in vivo transglutaminase assay] to more than 50%, and a cosmetically acceptable vehicle.

The boosters included in the inventive compositions are selected from the group consisting of:

- (a) Two boosters, wherein both are selected from the same group consisting of B2; B3; B4;

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(b) binary combinations of boosters selected from the group consisting of:

B1/B2; B1/B3; B1/B4; B1/B5; B2/B3, B2/B4; B2/B5, B3/B4; B3/B5;  
B4/B5;

(c) ternary combinations of boosters selected from the group consisting of:

B1/B2/B3; B1/B2/B4; B1/B2/B5; B1/B3/B4; B1/B3/B5;  
B1/B4/B5; B2/B3/B4; B2/B3/B5; B2/B4/B5; B3/B4/B5;

(d) quaternary combinations of boosters selected from the group consisting of:

B1/B2/B3/B4; B1/B2/B3/B5; B1/B2/B4/B5; B1/B3/B4/B5; B2/B3/B4/B5;  
and

(e) a combination of five groups of boosters: B1/B2/B3/B4/B5.

The preferred compositions include at least one booster from the different groups (i.e., groups (b) through (e) above). However, any combination of boosters chosen from the different groups may also be employed in the inventive compositions for desired boosting effects.

The compounds included in the present invention as boosters are first selected based on the ability of such compounds to pass, at a certain concentration listed in Table A, an in-vitro Microsomal Assay for a specific enzyme as described below under sections 2.1 through 2.7. The compound (alone or in combination with another booster) is then subjected to an in vitro transglutaminase assay described below, at an individual or combined concentration of 10 mM. If such combination inhibits transglutaminase to more than 50%, then it is suitable for use in the present invention. If a booster was tested individually, and passes the transglutaminase assay, then it

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may be combined with another booster or combination that passes the transglutaminase assay.

Preferred compositions according to the present invention contain  
5 combinations of boosters which at an individual concentration of 10 mM inhibit transglutaminase to more than 50%.

The term "conditioning" as used herein means prevention and treatment of dry skin, acne, photodamaged skin, appearance of wrinkles, age spots, aged skin,  
10 increasing stratum corneum flexibility, lightening skin color, controlling sebum excretion and generally increasing the quality of skin. The composition may be used to improve skin desquamation and epidermal differentiation.

A booster is a compound which passes an in vitro Microsomal Assay described  
15 below in sections 2.1 through 2.7. A compound suitable for use in the present invention inhibits or enhances, at a concentration listed in Table A an enzyme, to at least a broad % listed in Table A.

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TABLE A

Booster Test Concentrations and % Inhibition/Increase

ARAT / LRAT Assay *(To identify B1 boosters)*

Invention	Compound Concentration	% Inhibition
Broad	100 µM	> 10%
Preferred	100 µM	> 25%
Most Preferred	100 µM	> 40%
Optimum	100 µM	> 50%

Retinol Dehydrogenase Assay *(To identify B2 boosters)*

Invention	Compound Concentration	% Increase
Broad	100 µM	> 10%
Preferred	100 µM	> 15%
Most Preferred	100 µM	> 20%
Optimum	100 µM	> 25%

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Retinal Reductase Assay *(To identify B3 boosters)*

Invention	Compound Concentration	% Inhibition
Broad	100 µM	> 5%
Preferred	100 µM	> 10%
Most Preferred	100 µM	> 20%
Optimum	100 µM	> 35%

CRABPII Antagonist Assay *(To identify B4 boosters)*

Invention	Compound : RA Ratio	% Inhibition
Broad	7000 : 1	> 25%
Preferred	7000 : 1	> 50%
Most Preferred	70 : 1	> 25%
Optimum	70 : 1	> 50%

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**Retinoic Acid Oxidation Assay (To identify B5 boosters)**

Invention	Compound Concentration	% Inhibition
Broad	100 $\mu$ M	> 25%
Preferred	100 $\mu$ M	> 45%
Most Preferred	100 $\mu$ M	> 70%
Optimum	100 $\mu$ M	> 80%

The in vitro Microsomal Assays employed for determining the suitability of the inclusion of the compound in the inventive compositions are as follows:

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**1. Materials**

All-trans-retinol, all-trans-retinoic acid, palmitoyl-CoA, dilauroyl phosphatidyl choline, NAD, and NADPH were purchased from Sigma Chemical Company. Stock solutions of retinoids for the microsomal assays were made up in HPLC grade acetonitrile. All retinoid standard stock solutions for HPLC analysis were prepared in ethanol, stored under atmosphere of N<sub>2</sub> at -70°C and maintained on ice under amber lighting when out of storage. Other chemicals and the inhibitors were commercially available from cosmetic material suppliers or chemical companies such as Aldrich or International Flavors and Fragrances.

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**2. Methods**

2.1 Isolation of RPE microsomes (modified from J.C. Saari & D.L. Bredberg, "CoA and Non-CoA Dependent Retinol Esterification in Retinal Pigment Epithelium", J. Biol. Chem. 263, 8084-8090 (1988)).

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50 frozen hemisected bovine eyecups, with the retina and aqueous humor removed were obtained from W. L. Lawson Co., Lincoln, NE, USA. The eyes were thawed

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overnight and the colored iridescent membrane was removed by peeling with forceps. Each eyecup was washed with 2x 0.5mL cold buffer (0.1M PO<sub>4</sub> / 1mM DTT / 0.25M sucrose, pH 7) by rubbing the darkly pigmented cells with an artist's brush or a rubber policeman. The cell suspension was added to the iridescent membranes and the suspension was stirred for several minutes in a beaker with a Teflon stir bar. The suspension was filtered through a coarse filter (Spectra / Por 925μ pore size polyethylene mesh) to remove large particles, and the resulting darkly colored suspension was homogenized using a Glas-Col with a motor driven Teflon homogenizer. The cell homogenate was centrifuged for 30 min. at 20,000g (Sorvaal model RC-5B centrifuge with an SS34 rotor in 2.5x10cm tubes at 14,000 RPM). The resulting supernatant was subjected to further centrifugation for 60 min. at 150,000g (Beckman model L80 Ultracentrifuge with an SW50.1 rotor in 13x51mm tubes at 40,000 RPM). The resulting pellets were dispersed into ~5mL 0.1M PO<sub>4</sub> / 5mM DTT, pH 7 buffer using a Heat Systems Ultrasonics, Inc. model W185D Sonifier Cell Disruptor, and the resulting microsomal dispersion was aliquoted into small tubes and stored at -70°C. The protein concentrations of the microsomes were determined using the BioRad Dye binding assay, using BSA as a standard.

2.2 Isolation of rat liver microsomes (modified from R. Martini & M. Murray, "Participation of P450 3A Enzymes in Rat Hepatic Microsomal Renitoic Acid 4-Hydroxylation", Archives Biochem. Biophys. 303, 57-66 (1993)).

Approximately 6 grams of frozen rat liver (obtained from Harlan Sprague Dawley rats from Accurate Chemical and Scientific Corp.) were homogenized in 3 volumes of 0.1M tris / 0.1M KCl / 1mM EDTA / 0.25M sucrose, pH 7.4 buffer using a Brinkmann Polytron. The resulting tissue suspension was further homogenized in the motor driven Teflon homogenizer described above. The resulting homogenate was

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successively centrifuged for 30 min. at 10,000g, 30 min. at 20,000g, and 15 min. at 30,000g, and the resulting supernatant was ultracentrifuged for 80 min. at 105,000g. The pellet was sonicated in ~5mL of 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub> , pH 7.4 buffer as described above and stored as aliquots at -70°C. Protein concentrations  
5 were determined as described above.

**2.3 Assay for ARAT and LRAT activity (To identify B1)**

The procedure below is a modification of a method described in J.C. Saari & D.L. Bredberg, "ARAT & LRAT Activities of Bovine Retinal Pigment Epithelial Microsomes",  
10 Methods Enzymol. 190, 156-163 (1990). The following buffer was prepared and stored at 4°C: 0.1M PO<sub>4</sub> / 5mM dithiothreitol, pH 7.0 (PO<sub>4</sub> / DTT). On the day of the assay, add 2mg BSA per mL of buffer to give a PO<sub>4</sub> / DTT / BSA working buffer. 1mM retinol substrate was prepared in acetonitrile and stored in amber bottles under nitrogen gas at -20°C. Solutions of 4mM Palmitoyl-CoA in working buffer (stored in aliquots) and  
15 4mM dilauroyl phosphatidyl choline in ethanol were prepared and stored at -20°C. Inhibitors were prepared as 10mM stock solutions in H<sub>2</sub>O, ethanol, acetonitrile or DMSO. The quench solution was prepared using pure ethanol containing 50µg/mL butylated hydroxytoluene (BHT), and a hexane solution containing 50µg/mL BHT was used for the extractions.

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To a 2 dram glass vial, add the following in order: PO<sub>4</sub> / DTT / BSA buffer to give a total volume of 500µL, 5µL acyl donor (4mM palmitoyl-CoA and/or dilauroyl phosphatidyl choline), 5µL inhibitor or solvent blank (10mM stock or further dilutions) followed by approximately 15µg of RPE microsomal protein (approximately 15µL of a  
25 ~1mg/mL microsomal protein aliquot). Incubate for 5 min. at 37°C to equilibrate the reaction temperature and then add 5µL 1mM retinol. Cap the vials, vortex for 5 seconds and incubate for 30-90 minutes at 37°C. Quench the reaction by adding

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0.5mL ethanol / BHT. Extract the retinoids by adding 3mL hexane / BHT, vortex the tubes for several seconds several times and centrifuge the tubes at low speed for 5 min. to quickly separate the layers. Remove the upper hexane layer into a clean vial, and re-extract the aqueous layer with another 3mL hexane / BHT, as described  
5 above. Combine the hexane layers and evaporate the hexane by drying at 37°C under a stream of nitrogen gas on a heated aluminum block. Store the dried residue at -20°C until HPLC analysis. Quantitate the amount of retinyl palmitate and retinyl laurate for ARAT and LRAT activity, respectively, by integration of the HPLC signal as described below.

10 Note that the incubation solution contains 40µM acyl donor, 100µM or less inhibitor, 10µM retinol, approximately 30µg/mL microsomal protein, and nearly 0.1M PO<sub>4</sub>, pH 7 / 5mM DTT / 2mg/mL BSA. All steps subsequent to the addition of retinol were done in the dark or under amber lights.

15 **2.4 Assay for Retinol Dehydrogenase Activity** (To identify B2)

The following stock solutions were prepared:

20 50mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 buffer, sterile filtered.

10mM all trans Retinol (Sigma R7632) in DMSO.

200mM Nicotinamide adenine dinucleotide phosphate, sodium salt (NADP) (Sigma N0505) in sterile water.

40mM test compound in appropriate solvent (water, buffer, ethanol, chloroform or  
25 DMSO).

1:10 dilution of rat liver Microsomes in 50mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 buffer (4ug/ul).

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In a two-dram glass vial with screw cap, add the following in order:

Buffer to give a final volume of 400 $\mu$ l

- 25 $\mu$ l diluted Microsomes (final = 100ug) – use boiled Microsomes for controls and  
5 regular Microsomes for test samples.

4 $\mu$ l of 200mM NADP (final = 2mM)

1 $\mu$ l of 40mM test compound (final = 100 $\mu$ M)

8 $\mu$ l of 10mM retinol (final = 200 $\mu$ M)

- 10 Incubate vials in a 37°C shaking water bath for 45 minutes. Add 500 $\mu$ l ice-cold ethanol to each vial to quench the reaction. Extract the retinoids twice with ice cold hexane (2.7ml per extraction). Retinyl acetate (5 $\mu$ l of a 900 $\mu$ M stock) is added to each vial during the first extraction as a means of monitoring the extraction efficiency in each sample. Samples were vortexed for ten seconds before gently centrifuging for  
15 five minutes at 1000rpm, 5°C in a Beckman GS-6R centrifuge. The top hexane layer containing the retinoids is removed from the aqueous layer after each extraction to a clean two-dram vial. Evaporate off the hexane under a gentle stream of nitrogen gas. Store the dried residue at –20°C until HPLC analysis.

- 20 2.5 Assay for Retinal Reductase Activity (To identify B3)

All stock solution were prepared as above with the following substitutions:

10mM all trans Retinaldehyde (Sigma R2500) in DMSO – instead of retinol.

- 25 200mM, Nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt (NADPH) (Sigma N7505) in sterile water – instead of NADP.

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In a two-dram glass vial with screw cap, add the following in order:

Buffer to give a final volume of 400 $\mu$ l

- 25 $\mu$ l diluted Microsomes (final = 100 $\mu$ g) – use boiled Microsomes for controls and  
5 regular Microsomes for test samples.

4 $\mu$ l of 200mM NADPH (final = 2mM)

1 $\mu$ l of 40mM test compound (final = 100 $\mu$ M)

3 $\mu$ l of 10mM retinaldehyde (final = 75 $\mu$ M)

- 10 Follow the same incubation and extraction procedure as detailed above.

**2.6 Assay for CRABP II antagonists (To identify B4)**

**2.6.1 Synthesis of CRABP II**

- 15 a. System of expression

The gene CRABP II was cloned in pET 29a-c(+) plasmid (Novagen). The cloned gene was under control of strong bacteriophage T7 transcription and translation signals. The source of T7 polymerase was provided by the host cell E.coli BLR(DE3)pLysS (Novagen). The latter has a chromosomal copy of T7 polymerase under lacUV5  
20 control, induced by the presence of IPTG. The plasmid was transferred into E. coli BLR(DE3)pLysS cells by transformation according to the manufacturer protocol (Novagen).

**b. Induction**

- 25 An overnight culture of the transformed cells was diluted 1:100 into 2xYT containing 50  $\mu$ g/mL kanamycin and 25 $\mu$ g/mL chloramphenicol. The cells grew while shaking at 37°C until the OD at 600 nm reached 0.6-0.8. Then IPTG was added to a final

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concentration of 1mM and the culture was incubated for an additional two hours. The cells were harvested by centrifugation at 5000g for 10 minutes at room temperature. The pellet was stored at -20°C.

5    2.6.2 Purification

Purification was performed according to the method described in A. W. Norris and E. Li, "Generation and characterization of cellular retinoic acid-binding proteins from *Escherichia coli* expression systems. *Methods Enzymol*, 1997;282:3-13.

a. Lysis

10    The frozen pellet was thawed at RT and resuspended in 1-2 pellet volumes of freshly prepared lysis buffer (50 mM Tris-HCl, pH 8, 10%(w/v) sucrose, 1 mM EDTA, 0.05%(w/v) sodium azide, 0.5 mM DTT, 10 mM MnCl<sub>2</sub>, 2.5 mM phenylmethylsulfonyl fluoride, 2.5 mM benzamidine, 6µg/mL DNase). The lysate was incubated for 30 min at room temperature. Further lysis was accomplished by sonication (six 30-sec bursts at 10,000 psi alternated with five 30-sec delay on ice). The insoluble fraction of the  
15    lysate was removed by centrifugation at 15000 rpm 1 hour at 4°C and the supernatant is stored at -20°C.

b. Gel filtration on Sephacryl S300

20    The supernatant from step a. was loaded onto a 2.5x100 cm column of sephacryl S-300 (Pharmacia) at room temperature. The elution buffer was 20 mM Tris-HCl, pH 8, 0.5mM DTT, 0.05% sodium azide (buffer A). The flow rate was 2mL/min. Collected 2-mL fractions were checked for ultraviolet absorbance at 280 nm. The fractions representing the peaks were examined by SDS-page for the presence of CRABP II .

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c. Anion-exchange chromatography

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2 mL of gel filtration fractions containing CRABP II were loaded onto a quaternary amine anion-exchange column FPLC (Fast Protein Liquid Chromatography) type monoQ (Pharmacia). CRABP II was eluted using a gradient buffer from 100% buffer A to 30% buffer B (100 % buffer B = buffer A + 250 mM NaCl) over a 20-min period at room temperature. 1 mL-fractions were collected every minute. Once more, the presence of CRABP II was checked by SDS page. CRABP II was stored at 4°C before freeze-drying using a Micromodulyo 1.5K with vial platform attachment (Edwards High Vacuum International). The desiccated samples were stored at room temperature until their use in the binding assay.

d. Detection of the presence of CRABP II

The expression and purification of CRABP II was validated using denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 7-15% polyacrylamide gel (Biorad). 10 µL samples were mixed with 10 µL of 2X loading buffer (100 mM Tris-HCl pH6.8, 4% SDS, 0.2% BPB, 20% glycerol, 1mM DTT) and denatured by heating (2 min at 80°C). The samples were loaded onto the gel that was immersed in a 1X Tris-glycine buffer (Biorad) and a constant current (25 mA) was applied for 1 hour at room temperature. After Coomassie blue staining, the protein was identified according to its molecular weight as determined with the Benchmark prestained protein ladder (Gibco BRL).

A western blot was used to confirm the presence of CRABP II. The proteins separated on the SDS-PAGE were transferred on an Immobilon-P transfer membrane (Millipore) using a Biorad cassette. The transfer occurred in 1X Tris-glycine buffer (Biorad) + 10% methanol. An electrical current (60 mA) was applied for 3 hours to allow the protein to migrate through the membrane. Afterwards, the membrane was blocked with 5% dry milk in 1X TBS for one hour at room temperature and probed with primary antibodies

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to CRABP II (1/1000 dilution of mouse anticonal 5-CRA-B3) in the same buffer at 4°C overnight. The following day, the membrane was washed with PBS (3 x 5 minutes) and then incubated with 1:2000 dilution of the secondary antibody, peroxidase conjugated anti-mouse antibody (ECLTM, Amersham), for 1 hour at room temperature. The membrane was washed with 1xPBS (3x 5 minutes) and the protein was detected using ECL detection kit according to the manufacturer instruction (Amersham).

The concentration of purified CRABP II was determined using BSA kit (Pierce).

10    2.6.3 Radioactive Binding assay

220 pmol of CRABP II was incubated in 20 mM Tris-HCl buffer pH 7.4 with 15 pmol of radioactive all trans retinoic acid (NEN) in a total volume of 70µL. For the competitive assay, another ligand in excess (6670:1, 670:1 or 70:1) was added to the mix. The reaction occurred for one hour at room temperature in the dark. In order to separate the unbound all-trans retinoic acid from the bound all-trans retinoic acid, a 6kD cut-off minichromatography column (Biorad) was used. The storage buffer was discarded using a Microplex manifold for according to the manufacturer instruction (Pharmacia). The samples were loaded onto the column and the separation occurred by gravity over a 30-min period. Retinoic acid ("RA") bound to CRABP II appeared in the filtrate while free RA remained in the column. The radioactivity of the filtrate was measured by scintillation counter.

25    2.7 Assay for NADPH dependent retinoic acid oxidation (To identify B5)

The procedure below is a modification of a method described in R. Martini & M. Murray, "Participation of P450 3A Enzymes in Rat Hepatic Microsomal Retinoic Acid

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4-Hydroxylation", Archives Biochem. Biophys. 303, 57-66 (1993). Prepare the following assay buffer and store at 4°C: 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub>, pH 7.4. On the day of the assay, prepare a 60mM NADPH solution in buffer. Prepare inhibitor stocks, acidified ethanol / BHT quench solution, and hexane / BHT as described above. A working 1mM retinoic acid solution was prepared by dilution of a 15mM stock (in DMSO) with ethanol.

To a 2 dram vial, add the following in order: assay buffer to give a final volume of 500μL, 20μL 60mM NADPH, 5μL inhibitor or solvent blank, followed by approximately 2mg of rat liver microsomal protein.

Incubate for 5 min. at 37°C, then add 5μL working 1mM retinoic acid solution. Continue incubation for 60min. at 37°C - do not cap the vials, since the oxidation process requires molecular O<sub>2</sub> in addition to NADPH. Quench with acidified ethanol / BHT and extract with hexane / BHT as described above. Quantitate the quickly eluting polar retinoic acid metabolites (presumed to be 4-oxo retinoic acid) by integration of the HPLC signal, as described below.

Note that all steps subsequent to the addition of retinoic acid were done in the dark or under amber lights. The final incubation solution contains 2.4mM NADPH, 100μM or less inhibitor, 10μM retinoic acid, approximately 4mg/mL rat liver microsomal protein and nearly 0.1M PO<sub>4</sub> / 0.1mM EDTA / 5mM MgCl<sub>2</sub>.

**HPLC analysis of individual retinoids**

Samples for retinoid quantitation by HPLC were prepared by dissolving the residue in each vial with 100μL of methanol. The solution was transferred to a 150μL glass conical tube within a 1mL shell vial, capped tightly, and placed inside a Waters 715

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Autosampler. Aliquots of 60µL were injected immediately and analyzed for retinoid content.

5 The chromatography instrumentation consisted of a Waters 600 gradient controller / pump, a Waters 996 Photodiode Array detector and a Waters 474 Scanning Fluorescence detector. Two HPLC protocols were used for retinoid analysis. For the ARAT and LRAT assay, the separation of retinol and retinol esters was performed with a Waters 3.9x300mm C18 Novapak reverse-phase analytical column and Waters Sentry NovaPak C18 guard column with an 80:20(v/v) methanol / THF isocratic mobile  
10 phase adjusted to a flow rate of 1mL/min. for 10 min. The eluate was monitored for absorbance at 325nm and fluorescence at 325ex/480em. A shorter Waters 3.9x150mm C18 Novapak reverse-phase analytical column and Waters Sentry NovaPak C18 guard column were used to separate retinoid acids and alcohols for the retinol and retinoic acid oxidation assays utilizing a modification of a gradient system  
15 described by A. B. Barua, "Analysis of Water-Soluble Compounds: Glucuronides", Methods Enzymol. 189, 136-145 (1990). This system consisted of a 20 min. linear gradient from 68:32(v/v) methanol/ water containing 10mM ammonium acetate to 4:1(v/v) methanol:dichloromethane followed by a 5 min. hold at a flow rate of 1mL/min.. The column eluate was monitored from 300nm to 400nm.

20

These protocols were selected based on their ability to clearly resolve pertinent retinoid acids, alcohols, aldehydes, and/or esters for each assay and relative quickness of separation. Identification of individual retinoids by HPLC was based on an exact match of the retention time of unknown peaks with that of available authentic retinoid  
25 standards and UV spectra analysis (300-400nm) of unknown peaks against available authentic retinoids.

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The boosters suitable for further testing in the transglutaminase assay include but are not limited to the boosters listed in Tables B1 through B5 below.

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**ARAT/LRAT Inhibitors (B1)**

Class	Compound	% Inhibition Overall TG (- ROH/RE)	Overall TG (IC 50)	% Inhibition ARAT (10µm)	% Inhibition ARAT(100µm)	% Inhibition LRAT (10µm)	% Inhibition LRAT (100µm)
Carotenoid	Crocin		3.75E-05	15%	34%	0	15%
Fatty Acid Amides & Other Surfactants	Acetyl Sphingosine		6.78E-06	19%+/- 12	62% +/- 11	10% +/- 10	50% +/- 18
Fatty Acid Amides & Other Surfactants	C13 Beta-Hydroxy Acid/Amide	17%			28%		25%
Fatty Acid Amides & Other Surfactants	Castor Oil MEA		3.25E-05				
Fatty Acid Amides & Other Surfactants	Cocamidopropyl Betaine				25%		
Fatty Acid Amides & Other Surfactants	Coco Hydroxyethylimidazoline		2.84E-07		68%		68%
Fatty Acid Amides & Other Surfactants	Cocoamide-MEA (or Cocoyl Monoethanolamide)	11%			13%		34%
Fatty Acid Amides & Other Surfactants	Glycerol-PCA-Oleate				41% +/- 6		58% +/- 2
Fatty Acid Amides & Other Surfactants	Hexanoamide				20%		
Fatty Acid Amides & Other Surfactants	Hexanoyl Sphingosine		9.99E-05		28% +/-4		37% +/-9
Fatty Acid Amides & Other Surfactants	Hydroxyethyl-2-Hydroxy-C12 Amide		3.29E-05		35%		35%
Fatty Acid Amides & Other Surfactants	Hydroxyethyl-2-Hydroxy-C16 Amide				25%		30%
Fatty Acid Amides & Other Surfactants	Lauroyl Sarcosine				20%		
Fatty Acid Amides & Other Surfactants	Lidocaine				12%		0
Fatty Acid Amides & Other Surfactants	Linoleamide-DEA (or Linoleoyl Diethanolamide)	59%		12% +/-13	43% +/-3	11% +/-9	51% +/- 15
Fatty Acid Amides & Other Surfactants	Linoleamide-MEA (or Linoleoyl Monoethanolamide)		1.61E-05	14%	35%	20% +/- 8	35%
Fatty Acid Amides & Other Surfactants	Linoleamidopropyl Dimethylamine				69% +/-18		75% +/-4
Fatty Acid Amides & Other Surfactants	Melinamide				64% +/-15		43% +/-2 21
Fatty Acid Amides & Other Surfactants	Myristoyl Sarcosine				41% +/- 14		11% +/-11

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Class	Compound	% Inhibition Overall TG (- ROH/RE)	Overall TG (IC 50)	% Inhibition ARAT (10µm)	% Inhibition ARAT(100µm)	% Inhibition LRAT (10µm)	% Inhibition LRAT (100µm)
Fatty Acid Amides & Other Surfactants	Oleyl Betaine		2.80E-05	47%			
Fatty Acid Amides & Other Surfactants	Palmitamide-MEA			6%	23%	12%	33%
Fatty Acid Amides & Other Surfactants	Stearylhydroxyamide				10%		10%
Fatty Acid Amides & Other Surfactants	Utrecht-1	21%		43%	54%	51%	48% +/- 6
Fatty Acid Amides & Other Surfactants	Utrecht-2		3.47E-06	42%	83% +/-9	51%	92% +/-3
Flavonoids	Naringenin				33%		14%
Fragrances	Allyl Alpha-Ionone			16% +/-14	22% +/-23	17% +/- 10	36%/- 7
Fragrances	Alpha-Damascone		3.35E-04	67% +/- 27	83% +/- 12	87% +/- 6	98% +/- 1
Fragrances	Alpha=Ionone		9.27E-04		45% +/- 27		49% +/- 30
Fragrances	Alpha-Methyl Ionone				67%		77%
Fragrances	Alpha-Terpineol				26%		25%
Fragrances	Beta-Damascone			45%	84%	52%	92%
Fragrances	Brahmanol				70%		75%
Fragrances	Damascenone			23%	70%	29%	79%
Fragrances	Delta-Damascone			58%	87%	64%	95%
Fragrances	Dihydro Alpha-Ionone				13%		18%
Fragrances	Ethyl Safranate				51%		49%
Fragrances	Fenchyl Alcohol				12%		4%
Fragrances	Gamma-Methyl Ionone				21%		38%
Fragrances	Isobutyl Ionone				8%		45%
Fragrances	Isocyclogeraniol				18%		16%
Fragrances	Isodamascone				80%		92%

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Class	Compound	% Inhibition Overall TG (- ROH/RE)	Overall TG (IC 50)	% Inhibition ARAT (10µm)	% Inhibition ARAT (100µm)	% Inhibition LRAT (10µm)	% Inhibition LRAT (100µm)
Fragrances	Lyrall		1.27E-04	76%	71%		
Fragrances	Santalone			23%	12%		
Fragrances	Santanol			15%	43%		
Fragrances	Timberol			34%	33%		
Fragrances	Tonalid			50%	33%		
Fragrances	Traseolide			41%	21%		
Miscellaneous	Coco Trimethylammonium Cl-			27%			
Miscellaneous	Urosolic Acid		1.46E-06	21%	28%		
Noncyclic Fragrances	Citral			20%			
Noncyclic Fragrances	Citronellol			30%	0		
Noncyclic Fragrances	Farnesol		9.35E-05	23%+/- 18	53% +/- 18	10% +/- 7	53% +/- 19
Noncyclic Fragrances	Geraniol		7.83E-03	13%	32%		
Noncyclic Fragrances	Geranyl Geraniol			38% +/- 12	81% +/- 6	16% +/- 9	77% +/- 13
Noncyclic Fragrances	Linatool				28%		0
Noncyclic Fragrances	Nonadieneal				20%		
Noncyclic Fragrances	Pseudoionone				12%		37%
Phospholipid	Diocetylphosphatidyl Ethanolamine						
Urea	Dimethyl Imidazolidinone	22%		23%	50% +/- 2	0	17% +/- 17
Urea	Imidazolidinyl Urea	35%					

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### Retinol Dehydrogenase Activators (B2)

Class	Compound	% Increase Retinol Dehydrogenase
Phospholipid	Phosphatidyl Choline	21% increase
Phospholipid	Sphingomyelin	26% increase

### Retinaldehyde Reductase Inhibitors (B3)

Class	Compound	Overall TG (IC 50)	% Inhibition Retinal Reductase
Aldehyde	Vanillin	9.70E-03	6%
Fatty Acid	Arachidic Acid		20%
Fatty Acid	Arachidic Acid		49%
Fatty Acid	Linoleic Acid	1.63E-04	62% +/-2
Fatty Acid	Linolenic Acid	1.34E-04	54% +/-16
Fatty Acid	Myristic Acid	1.72E-05	26%
Miscellaneous	Amsacrine	6.26E-06	22% + /-8
Miscellaneous	Carbenoxolone	3.61E-07	26% +/-2
Miscellaneous	Glycyrrhetic Acid	8.64E-06	38% +/- 1
Phospholipid	Phosphatidyl ethanolamine		37%

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CRABPII Antagonists (B4)

Class	Compound	Overall TG (IC 50)	% Inhibition CRABPII
Fatty Acid	Elaidic Acid	6.50E-05	>50%
Fatty Acid	Hexadecanedioic Acid	1.30E-04	>50%
Fatty Acid	12-Hydroxystearic Acid	2.91E-05	>50%
Fatty Acid	Isostearic Acid	6.88E-05	>50%
Fatty Acids	Linseed Oil		>50%

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Retinoic Acid Oxidation Inhibitors (B5)

Class	Compound	Overall TG (IC 50)	% Inhibition Retinoic Acid (10µM)	% Inhibition Retinoic Acid (100µM)
Imidazole	Bifonazole		89%	100%
Imidazole	Climbazole	4.47E-06	80%	92%
Imidazole	Clotrimazole		76%	85%
Imidazole	Econazole		88%	100%
Imidazole	Ketoconazole	1.85E-07	84%	84%
Imidazole	Miconazole	2.78E-07	74%	86%
Fatty Acid Amides & Other Sufactants	Lauryl Hydroxyethylimidazoline	4.67E-07		
Fatty Acid Amides & Other Sufactants	Oleyl Hydroxyethylimidazoline	3.02E-05	54%	80%
Flavanoids	Quercetin	6.29E-05	40%	74%
Coumarin	Coumarin			
Quinoline	(7H-Benzimidazo[2, 1-a]Benz[de]-Isoquinolin-7-one	8.59E-07		
Quinoline	Hydroxyquinoline (Carbostyrl)	3.64E-04		
Quinoline	Metrapone (2-Methyl-1, 2-di-3-Pyridyl-1-Propane			47%

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The boosters or combinations thereof inhibit transglutaminase (hereinafter "Tgase") in a transglutaminase assay described below to at least 50% at a concentration of 10mM.

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TGase Assay		
Invention	Compound Concentration	% Inhibition
Broad	10 mM	> 50%
Preferred	1 mM	> 50%
Most Preferred	100 $\mu$ M	> 50%
Optimum	10 $\mu$ M	> 50%

### Transglutaminase Assay and Keratinocyte Differentiation

10 During the process of terminal differentiation in the epidermis, a 15nm thick layer of protein, known as the cornified envelope (CE) is formed on the inner surface of the cell periphery. The CE is composed of numerous distinct proteins which have been cross-linked together by the formation of N<sup>ε</sup>-( $\gamma$ -glutamyl) lysine isodipeptide bonds catalyzed by the action of at least two different transglutaminases

15 (TGases) expressed in the epidermis. TGase I is expressed in abundance in the differentiated layers of the epidermis, especially the granular layer, but is absent in the undifferentiated basal epidermis. Thus TGase I is a useful marker of epidermal keratinocyte differentiation with high TGase I levels indicating a more differentiated state. An ELISA based TGase I assay, using a TGase I antibody, was used to assess

20 the state of differentiation of the cultured keratinocytes in the examples that follow.

Keratinocytes (cultured as described above) were plated in 96 well plates at a density of 4,000-5,000 cells per well in 200 $\mu$ l media. After incubation for

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two to three days, or until cells are ~50% confluent, the media was changed to media containing test compounds (five replicates per test). The cells were cultured for a further 96 hours after which time the media was aspirated and the plates stored at -70°C. Plates were removed from the freezer, and the cells were washed twice  
5 with 200µl of 1x PBS. The cells were incubated for one hour at room temperature (R/T) with TBS/5% BSA (wash buffer, bovine serum albumin). Next the TGase primary antibody was added: 50µl of monoclonal anti-Tgase I Ab B.C. diluted 1:2000 in wash buffer. The primary antibody was incubated for 2 hours at 37°C and then rinsed 6x with wash buffer. Cells were then incubated with 50µl of secondary  
10 antibody (Fab fragment, peroxidase conjugated anti-mouse IgG obtaining from Amersham) diluted 1:4,000 in wash buffer for two hours at 37°C, then rinsed three times with wash buffer. Following the rinse with washing buffer, the cells were rinsed 3x with PBS. For colourimetric development, the cells were incubated with  
15 100µl substrate solution (4 mg o-phenylenediamine and 3.3 µl 30% H<sub>2</sub>O<sub>2</sub> in 10ml 0.1M citrate buffer pH 5.0) for exactly five minutes, R/T, in darkness (under aluminum foil). The reaction was stopped by the addition of 50µl 4N H<sub>2</sub>SO<sub>4</sub>. The absorbance of samples was read at 492nm in a 96 well plate UV spectrophotometer. Out of the five replicates, four were treated with both antibodies, the fifth one was  
20 use as a Tgase background control. TGase levels were determined and expressed as percentage control.

Transglutaminase levels were determined and expressed in the Tables B1 through B5 above either as:

(i) % (booster + retinol inhibition / control inhibition) - % (ROH inhibition / control inhibition), which measures the added effect of booster + retinol induced TGase inhibition over retinol alone, or

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(ii) as an IC<sub>50</sub> value when the inhibitory effect of multiple booster concentrations was examined - this provides the concentration of booster which, in combination with a constant retinol concentration of 10<sup>-7</sup>M, inhibits TGase by 50%.

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It is the IC<sub>50</sub> value that is used as a benchmark in the present invention.

**Best Groups of Boosters for testing in Transglutaminase assay**

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**B1 Compounds**

1. Fatty Acid Amides	These are readily commercially available and have the added advantage of being surfactants and thus help generate emulsions suitable for cosmetic preparations.
2. Ceramides	These can additionally act as precursors of stratum corneum barrier ceramides.
3. Carotenoids	These can offer some UV protection and and act as natural colorants.
4. Flavanoids	Natural antioxidants.
5. Cyclic fragrances	These are readily commercially available and additionally can be used to fragrance the product.
6. Non-cyclic fragrances	These can be used to fragrance the product.
7. Phospholipid analogues	These can be utilised by skin cells to nourish the generation of barrier components.
8. Ureas	These are readily commercially available and can also act as preservatives for the product.

15 **B2 Compounds**

1. Phosphatidyl choline	Most preferred as most active activator of Retinol Dehydrogenase
2. Sphingomyelin	

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### B3 Compounds

Arachidonic Acid Linoleic Acid Linolenic Acid Myristic Acid	Fatty Acids which can be useful in maintaining stratum corneum barrier
Linoleic Acid Linolenic Acid	Essential Fatty Acids
Arachidonic Acid Myristic Acid	Non-essential fatty acids
Glycyrrhetic Acid	Polycyclic triterpene carboxylic acid which is readily obtained from plant sources.
Phosphatidyl ethanolamine	Can be incorporated into cellular membranes.

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### B4 Compounds

Hexadecanedioic acid 12-hydroxystearic acid Isostearic acid	Saturated fatty acids.
Linseed oil Elaidic acid	Unsaturated fatty acids
Elaidic acid Isostearic acid Hexadecanedioic acid	Solid at room temperature
Linseed oil 12-hydroxystearic acid	Liquid at room temperature

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## B5 Compounds

Bifonazole Climbazole Clotrimazole Econazole Ketoconazole Miconazole	Antimicrotics
Climbazole	Readily commercially available
Lauryl hydroxyethylimidazoline	Compounds which are readily commercially available and have the added advantage of being surfactants and thus help generate emulsions suitable for cosmetic preparations.
Quercetin	Naturally occurring flavanoid which has antioxidant properties.
Coumarin	Natural colorant
Quinolines Isoquinolines	
Metyrapone	

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## PHYTOESTROGEN

10 As part of the present invention, it has been surprisingly found that phytoestrogens synergistically improve the skin benefits of retinoids. Essentially, phytoestrogens increase the sensitivity of the skin to retinoids.

15 Therefore, the present invention contains about 0.001% to about 10% of at least one phytoestrogen in the second composition.

Phytoestrogens include flavonoids such as estrogenic flavonoids, genistein, daidzein, glycitin, biochanin A, formononetin and equol and mixtures thereof, acetyl

and malonyl esters of genistein and daidzein, and glucosides of genistein and daidzein. It should be noted that the aforementioned list is not exclusive, and may include other phytoestrogens known to persons of ordinary skill in the art.

5                   **DUAL COMPARTMENT PACKAGE**

10                   Compositions which include retinoids are generally unstable and may undergo chemical degradation. Moreover, it has been surprisingly found that boosters, although beneficial for enhancing the retinoid benefits, also contribute to the chemical instability of retinoids. The booster induced retinol destabilization dramatically reduces the overall efficacy of the boosted retinoid composition when both ingredients are contained in a single formula. Therefore, in order to protect against retinoid breakdown while still providing the beneficial effects of retinoid boosters, the present invention provides a dual compartment package that  
15                   contains a first composition containing retinoids in a first compartment and a second composition containing at least one retinoid booster in a second compartment. The first composition provides a first benefit to the skin while the second composition works to boost or enhance the effect of the first benefit.

20                   As a further retinoid enhancing benefit, phytoestrogens are an essential component of the present invention. Phytoestrogens such as genistein and daidzein synergistically interact with retinoids to deliver skin benefits. However, phytoestrogens contribute to the oxidation, and thus the degradation of retinoids. Therefore, the present invention provides the phytoestrogen as part of the second  
25                   composition in the second compartment of the dual compartment package, to further enhance the effect of the first benefit.

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The dual compartment package may be designed in various ways known to persons of ordinary skill in the art as long as the purpose of providing the first and second compositions in two separate containers is achieved. In one embodiment, the dual compartment package is in the form of two jars or bottles adjointly attached. In a second embodiment, the dual compartment package is in the form of a single bottle/jar with a division separating an interior of the bottle/jar into a first and second compartment. Other embodiments are contemplated as being within the scope of the present invention as long as the compositions are retained separately.

**Cosmetically Acceptable Vehicle**

The product according to the present invention also comprises a cosmetically acceptable vehicle to act as a dilutant, dispersant, or carrier for the active components in the either or both the first and second compositions, so as to facilitate their distribution when the composition is applied to the skin.

Vehicles other than or in addition to water can include liquid or solid emollients, solvents, humectants, thickeners and powders. An especially preferred nonaqueous carrier is a polydimethyl siloxane and/or a polydimethyl phenyl siloxane. Silicones of this invention may be those with viscosities ranging anywhere from about 10 to 10,000,000 centistokes at 25 °C. Especially desirable are mixtures of low and high viscosity silicones. These silicones are available from the General Electric Company under trademarks Vicasil, SE and SF and from the Dow Corning Company under the 200 and 550 Series. Amounts of silicone which can be utilized in the compositions of this invention range anywhere from 5 to 95%, preferably from 25 to 90% by weight of the composition.

### Optional Skin Benefit Materials and Cosmetic Adjuncts

5 In either one or both of the first and second compositions of the present invention, an oil or oily material may be present, together with an emulsifier to provide either a water-in-oil emulsion or an oil-in-water emulsion, depending largely on the average hydrophilic-lipophilic balance (HLB) of the emulsifier employed.

10 Various types of active ingredients may be present in either one or both of the first and second cosmetic compositions of the present invention and are described below. Actives are defined as skin or hair benefit agents other than emollients and other than ingredients that merely improve the physical characteristics of the composition. Although not limited to this category, general  
15 examples include sunscreens, skin lightening agents, tanning agents.

Sunscreens include those materials commonly employed to block ultraviolet light. Illustrative compounds are the derivatives of PABA, cinnamate and salicylate. For example, octyl methoxycinnamate and 2-hydroxy-4-methoxy  
20 benzophenone (also known as oxybenzone) can be used. Octyl methoxycinnamate and 2-hydroxy-4-methoxy benzophenone are commercially available under the trademarks, Parsol MCX and Benzophenone-3, respectively.

25 The exact amount of sunscreen employed in the emulsions can vary depending upon the degree of protection desired from the sun's UV radiation.

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Another preferred optional ingredient is selected from essential fatty acids (EFAs), i.e., those fatty acids which are essential for the plasma membrane formation of all cells. In keratinocytes EFA deficiency makes cells hyperproliferative. Supplementation of EFA corrects this. EFAs also enhance lipid biosynthesis of epidermis and provide lipids for the barrier formation of the epidermis. The essential fatty acids are preferably chosen from linoleic acid,  $\gamma$ -linolenic acid, homo- $\gamma$ -linolenic acid, columbinic acid, eicosa-(n-6,9,13)-trienoic acid, arachidonic acid,  $\gamma$ -linolenic acid, timnodonic acid, hexaenoic acid and mixtures thereof.

Emollients are often incorporated into cosmetic compositions of the present invention. Levels of such emollients may range from about 0.5% to about 50%, preferably between about 5% and 30% by weight of the total composition. Emollients may be classified under such general chemical categories as esters, fatty acids and alcohols, polyols and hydrocarbons.

Esters may be mono- or di-esters. Acceptable examples of fatty di-esters include dibutyl adipate, diethyl sebacate, diisopropyl dimerate, and dioctyl succinate. Acceptable branched chain fatty esters include 2-ethyl-hexyl myristate, isopropyl stearate and isostearyl palmitate. Acceptable tribasic acid esters include triisopropyl trilinoleate and trilauryl citrate. Acceptable straight chain fatty esters include lauryl palmitate, myristyl lactate, oleyl eurcate and stearyl oleate. Preferred esters include coco-caprylate/caprate (a blend of coco-caprylate and coco-caprate), propylene glycol myristyl ether acetate, diisopropyl adipate and cetyl octanoate.

Suitable fatty alcohols and acids include those compounds having from 10 to 20 carbon atoms. Especially preferred are such compounds such as cetyl, myristyl, palmitic and stearyl alcohols and acids.

Among the polyols which may serve as emollients are linear and branched chain alkyl polyhydroxyl compounds. For example, propylene glycol, sorbitol and glycerin are preferred. Also useful may be polymeric polyols such as polypropylene glycol and polyethylene glycol. Butylene and propylene glycol are also especially preferred as penetration enhancers.

Exemplary hydrocarbons which may serve as emollients are those having hydrocarbon chains anywhere from 12 to 30 carbon atoms. Specific examples include mineral oil, petroleum jelly, squalene and isoparaffins.

Another category of functional ingredients within the cosmetic compositions of the present invention are thickeners. A thickener will usually be present in amounts anywhere from 0.1 to 20% by weight, preferably from about 0.5% to 10% by weight of the composition. Exemplary thickeners are cross-linked polyacrylate materials available under the trademark Carbopol from the B.F. Goodrich Company. Gums may be employed such as xanthan, carrageenan, gelatin, karaya, pectin and locust beans gum. Under certain circumstances the thickening function may be accomplished by a material also serving as a silicone or emollient. For instance, silicone gums in excess of 10 centistokes and esters such as glycerol stearate have dual functionality.

Powders may be incorporated into one or both of the first and second cosmetic compositions of the cosmetic product of the present invention. These powders include chalk, talc, Fullers earth, kaolin, starch, smectite clays, chemically modified magnesium aluminum silicate, organically modified montmorillonite clay,

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hydrated aluminum silicate, fumed silica, aluminum starch octenyl succinate and mixtures thereof.

5 Other adjunct minor components may also be incorporated into one or both of the first and second compositions of the cosmetic product of the present invention. These ingredients may include coloring agents, opacifiers and perfumes. Amounts of these materials may range anywhere from 0.001% up to 20% by weight of the composition.

10 The first and second compositions of the cosmetic product of the present invention are intended primarily as a product for topical application to human skin, especially as an agent for conditioning and smoothening the skin, and preventing or reducing the appearance of wrinkled or aged skin.

15 In use, a small quantity of the first composition, for example from 1 to 5ml, is applied to exposed areas of the skin, from a suitable container or applicator and, if necessary, it is then spread over and/or rubbed into the skin using the hand or fingers or a suitable device. Simultaneously, a small quantity of the second composition, for example from 1 to 5 ml, is applied to exposed areas of the skin, 20 from a suitable container or applicator and, if necessary, it is also spread over and/or rubbed into the skin using the hand or fingers or a suitable device. Therefore, depending upon the intensity of treatment benefits desired, the first and second compositions may be used alone, simultaneously, or in consecutive order.

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**Product Form and Packaging**

The topical skin treatment composition of the invention can be formulated as a lotion, a fluid cream, a cream or a gel.

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**EXAMPLE 1**

**Methods**

10 Retinol (50% in tween 80) was dissolved in approximately 50% aqueous ethanol to provide a solution giving an OD at 360nm of approximately 0.6 when measured in a 200 µl volume in a 96 well plate using a standard 96 well spectrophotometer.

15 Booster molecules were added at approximately 0.1% concentration and the OD 360 measured as above immediately and after 60 hours at room temperature in the dark. A correction was applied to the OD after 60 hours (divide by 0.85) to account for increased concentration of the retinol due to evaporation of solvent from the plate.

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Results

TABLE 1

BOOSTER	FOLD INCREASE IN RATE OF RETINOL LOSS
CITRAL	3.1
CITRONELLOL	1.5
COCAMIDE DEA	1.9
COUMARIN	1.4
DAMASCONE	3.7
1,3 DIMETHYL 2 IMIDAZOLIDINONE	1.4
GERANIOL	1.3
18b GLYCERHETINIC ACID	1.6
8 OH QUINOLINE	1.5
N LAURY SARCOSINE	2.6
LINALOOL	2.0
LINOLEAMIDE DEA	3.0
LINOLEIC ACID	3.4
ALPHA IONONE	1.3
LINSEED OIL	1.5

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The Boosters tested caused marked increases in the instability of the retinol.

This will make it necessary to use formulation/packaging options providing considerably better stability to the retinol when boosters are used compared to those needed for retinol alone.

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EXAMPLE 2

To establish whether synergistic inhibition of transglutaminase expression occurred by combinations of B1 and B5 active compounds with retinol, it is essential to determine the dose response profiles (including IC50 values) of the active compounds when tested individually in the presence of retinol. This data was used to determine an appropriate sub-maximal inhibitory concentration of each active compound, to make it possible to identify synergistic effects of mixtures of the active compounds in the presence of retinol. In order to demonstrate synergy of two compounds, it is essential to select concentrations to test that are at most IC20, in other words a compound concentration that individually boosts the retinol inhibition of transglutaminase expression by 20%. Two such compounds should have an additive inhibition of 40%. Using this strategy to determine concentration leaves a window of 40-100% for further transglutaminase inhibition for detecting synergy of the two compounds under examination. A more challenging concentration criteria would be selecting concentrations of compounds which alone showed no boosted retinol inhibition of transglutaminase. In this study however we chose an even more challenging criteria. We selected concentrations of compounds that were 10 fold and 100 fold lower than the minimally effective transglutaminase inhibiting concentration. Identification of synergistic combinations using such very low concentrations would mean that the most effective synergistic combinations were identified.

The data in the following table represents the concentrations of compound that are 2 logs lower than the minimally inhibitory compound concentration. These were the concentrations used in the B1/B5 combination studies.

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**TABLE 2**

Compound	Concentration
<b>B1 Compounds</b>	
Linoleoyl monoethanolamide	1.00E-06
Palmitamide monoethanolamide	1.00E-06
Farnesol	3.16E-06
Hexyl sphingosine	1.00E-06
Utrecht-2	3.16E-08
Oleoyl betaine	3.16E-07
Oleoyl hydroxyethylimidazoline	1.00E-08
Cocoyl hydroxyethylimidazoline	1.00E-09
Ursolic acid	1.00E-08
Alpha-ionone	3.16E-05
<b>B5 Compounds</b>	
Ketoconazole	1.00E-09
Miconazole	3.16E-09
Climbazole	1.00E-08
Amino benzotriazole	1.00E-06
3,4-dihydroquinoline	1.00E-06
2-hydroxyquinoline	3.16E-06

To investigate synergistic inhibition of transglutaminase expression by combinations of B1 and B5 active compounds with retinol, selected combinations of compounds were tested at concentrations given in the above table. The following data was obtained:

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**TABLE 3**

Combination	B1 Compound	B5 Compound	Mean % control TGase
<b>B1 / B5</b>	Farnesol	Ketoconazole	84%
<b>B1 / B5</b>	Hexanoyl sphingosine	Miconazole	68%
<b>B1 / B5</b>	Hexanoyl sphingosine	Ketoconazole	64%
<b>B1 / B5</b>	Hexanoyl sphingosine	3,4-dihydroquinoline	89%
<b>B1 / B5</b>	Hexanoyl sphingosine	Aminobenzotriazole	81%
<b>B1 / B5</b>	Hexanoyl sphingosine	Climbazole	63%
<b>B1 / B5</b>	Oleoyl betaine	Ketoconazole	81%
<b>B1 / B5</b>	Oleoyl hydroxyethylimidazoline	Climbazole	52%
<b>B1 / B5</b>	Cocoyl hydroxyethylimidazoline	Climbazole	71%
<b>B1 / B5</b>	Ursolic acid	2-hydroxyquinoline	74%
<b>B1 / B5</b>	Alpha-ionone	Miconazole	84%
<b>B1 / B5</b>	Alpha-ionone	Ketoconazole	82%
<b>B1 / B5</b>	Alpha-ionone	2-hydroxyquinoline	76% ✓
<b>B1 / B5</b>	Utrecht-2	Aminobenzotriazole	82%
B1 / B5	Linoleoyl monoethanolamide	Ketoconazole	93%
B1 / B5	Linoleoyl monoethanolamide	Climbazole	94%
B1 / B5	Naringenin	Ketoconazole	100%
B1 / B5	Quercetin	Climbazole	92%
B1 / B5	Castor Oil monoethanolamide	Climbazole	98%
B1 / B5	Castor Oil monoethanolamide	Clotrimazole	100%

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The efficacy of the B1/B5 combinations splits into two classes – particularly effective combinations (bolded in the above table, i.e., the first fourteen combinations) and barely effective combinations (not bolded, i.e., the last six combinations). It was unexpected that certain B1/B5 combinations performed better than other combinations. Those combinations which were barely effective were (i) fatty acid amides + azoles (ii) hydroxy fatty acid amides + azoles and (iii) naringenin/quercetin + azoles. The effective combinations contained B1 boosters

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combined with B5 boosters from the following classes: fatty hydroxyethyl imidazoline surfactants, cyclic aliphatic unsaturated compounds, polycyclic triterpenes, n-substituted fatty acid amides.

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**EXAMPLE 3**

This example shows the synergy of retinoids and phytoestrogens:

(a) Cell culture method:

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Human adult fibroblasts obtained from sun-protected inner arm of 25-30 year female volunteer were used in this. Cells were grown in 1:1 DMEM/Hams F12 media containing 10% FBS, maintained at 37°C in a 5% CO<sub>2</sub> atmosphere under normal atmosphere oxygen tension. Third passage adult fibroblasts were grown in DMEM media with 10% FBS in 12-well plates at a seeding density of 2500 cells/ml/well. The cells at 80% confluence were rinsed in serum free and phenol red free (PRF) DMEM media twice. Pre-treatment with phyto-compounds for 4 hours was conducted and then dosed with retinoids and was incubated for 48 hours. After the incubation, the wells were washed twice with 1X PBS and the cell monolayer was harvested in 100 µl cell lysis buffer (contains 1X PBS, 1% Triton X, 0.5% sodium deoxycholate, 0.1% SDS containing protease inhibitor (10mg/ml PMSF in isopropanol, 10µl/ml). The suspension was spun at 14000rpm for 10 minutes, the supernatant collected and an aliquot of this supernatant was used for protein quantification. Protein concentration was determined using Pierce protein kit. The remainder of 100 µl supernatant (cell lysate) was denatured in a mixture of 40 µl sample buffer (NOVEX) and 0.5% Beta mercaptoethanol (BME) and by boiling the sample for 5 minutes. Equal amount of protein was then loaded onto

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16% Tris-glycine gels for protein analysis by SDS page and Western Immunoblotting for CRABP-2 protein expression.

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(b) Detection of Cellular Retinoic Acid Binding Protein II (CRABP-II):

Within the cells, retinol and retinoic acid are bound to specific cellular binding proteins, 2 of the major proteins are CRABP-I and II (Roos et al., Pharmacological reviews: 50, 315-333, 1998). These proteins act in regulating the intracellular concentration of retinoids by acting as both storage or shuttle proteins in retinoid metabolism. High or low levels of retinoids cause cell damage, including cell death, therefore regulation of constant levels of retinoids and its binding proteins are very critical for cell survival. The levels of this protein are regulated by the amount of retinoic acid within the cells. Higher cellular levels of retinoids increase the expression of CRABP-II. Therefore, the amount of this protein in the cells, is a measure of the retinoid activity of the cells. Skin cells contain high levels of CRABP-II both in the epidermis and the dermis. CRABP-II response to retinoid administration in fibroblasts in vitro is used as a reproducible measure of retinoid bioactivity that predict human skin responses. (Elder et al., J. Invest. Dermatol., 106: 517-521, 1996). Increase in CRABP-II is also associated with increased epidermal differentiation, and dermal retinoid action. Therefore, in these studies we used CRABP-2 expression of fibroblasts as a measure of retinoid activity leading to increased epidermal differentiation (skin conditioning and dry skin benefit) and dermal collagen and extracellular matrix synthesis (anti-aging, anti-wrinkling benefits).

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To measure the levels of CRABP-II in the fibroblasts, the equal amount of protein of cell supernatant were loaded onto to nitrocellulose blots in a dot blot apparatus as instructed by the manufacturer, and immunostaining was carried out using monoclonal antibodies to CRABP-II according to standard procedures. The CRABP-II protein band was visualized in the Dot Blots using the chemiluminescence system obtained from Santa Cruz Biotechnology (SantaCruz, CA). The bands in the film were quantitated by densitometric scanning, the data from the triplicate samples were calculated as % of control and expressed in the following tables as % increase over control (with control as 100%) +/-SD triplicates.

**EXAMPLE 4**

This example shows the stability of Retinol in the Presence of Phytoestrogenic Flavonoids.

Retinol was dissolved as a 10% solution in aqueous ethanol (1:1 water:ethanol). This solution was diluted to 0.001% approximately 30  $\mu$ M). This solution gave an OD of about 0.35 absorption unit at 360 nm in a 96 well plate spectrophotometer.

Aqueous ethanolic stock solutions of the genistein, daidzein were prepared as 0.1%, 0.01% or 0.001%. To 200  $\mu$ l of 0.001% retinol solution in a 96 well plate was added 20  $\mu$ l of the flavonoid (i.e. 1-10 dilution) giving a final flavonoid concentration of 0.01, 0.001 and 0.0001%. The plates were mixed and an initial OD reading was taken at 360 nm. The plates were incubated at room temperature in the dark for up to 2 days and subsequent readings were taken at

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8, 24 and 48 hours. The OD readings at these time points were normalized to the 0 time point reading. The retinol stability was expressed as % of retinol (OD reading) at 0 time. The data is shown in example 5.

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EXAMPLE 5

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In the 2 tables shown below, synergy between genistein and daidzein and retinoids were tested. In both the studies genistein was delivered to the cells in a soluble form in DMSO: ethanol. 1  $\mu$ M genistein alone stimulated CRABP-II significantly. Both genistein and daidzein stimulate retinoid activity in a synergistic manner. All the retinoids tested, except retinyl acetate showed synergy with genistein and daidzein. These data support the our claim that the phytoestrogenic flavonoids genistein and daidzein, when supplied to cells in a soluble form, synergistically enhanced the activity of retinoids.

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**TABLE 4**

Synergy between genistein and retinoids.

	CRABP-II production	% as Control	P value vs. Control p<0.05	P value vs. retinoids p<0.05	Synergy
Control	0.29+/-0.07	100+/-27	1		
10nM Retinoic acid	1.24+/-0.29	428+/-101	0.0055	1	
1nM Retinoic acid	0.97+/-0.47	335+/-162	0.068	1	
100nM Retinyl Linoleate	0.52+/-0.3	181+/-110	0.28	1	
100nM Retinyl Palmitate	1.26+/-0.51	434+/-177	0.032	1	
100nM Retinyl Acetate	0.60+/-0.32	209+/-118	0.19	1	
1µM Genistein	1.9+/-0.71	655+/-247	0.018		
1µM Genistein + 10nM Retinoic acid	4.18+/-0.31	1441+/-108	3.23E-05	2.96E-04	YES
1µM Genistein + 1nM Retinoic acid	4.01+/-0.61	1383+/-394	0.00049	0.012	YES
1µM Genistein + 100nM Retinyl linoleate	4.08+/-1.14	1408+/-213	0.0045	0.000982	YES
1µM Genistein + 100nM Retinyl palmitate	4.32+/-0.13	1489+/-47	160E-06	5.76E-04	YES
1µM Genistein + 100nM Retinyl acetate	2.32+/-0.91	800+/-313	1.80E-02	3.80E-02	NO

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**TABLE 5**

**Synergy Between Daidzein and Retinoids**

	CRABP-2 production	% as Control	P value vs. Control p<0.05	P value vs. retinoids p<0.05	Synergy
Control	0.29+/-0.07	100+/-27	1		
10nM Retinoic acid	1.24+/-0.29	428+/-101	0.0055	1	
1nM Retinoic acid	0.97+/-0.47	335+/-162	0.068	1	
100nM Retinyl Linoleate	0.52+/-0.3	181+/-110	0.28	1	
100nM Retinyl Palmitate	1.26+/-0.51	434+/-177	0.032	1	
100NM Retinyl Acetate	0.60+/-0.32	209+/-118	0.19	1	
1µM Daizedein	1.49+/-0.66	513+/-227	0.035		
1µM Daizedein + 10nM Retinoic acid	3.42+/-1.01	1181+/-350	0.0059	0.023	YES
1µM Daizedein + 1nM Retinoic acid	3.52+/-0.47	1213+/-163	0.000309	0.027	YES
1µM Daizedein + 100nM Retinyl linoleate	3.29+/-0.14	1136+/-142	0.00024	0.00078	YES
1µM Daizedein + 100nM Retinyl palmitate	2.51+/-0.19	865+/-65	4.90E-05	1.69E-02	YES
1uM Daizedein + 100nM Retinyl acetate	2.27+/-1.4	782+/-489	0.07	0.11	NO

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**EXAMPLE 6**

Example 5: The following tables show the effect of genistein and daidzein on destabilizing retinol. The experiment was done as described in methods section. The OD readings from duplicate measurements were averaged and given here.

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TABLE 6

Retinol stability in the presence of Genistein:

Time(hours)	0% Genistein	0.0001%	0.01%	0.01%
8	92	84	82	82
24	86	76	74	80
48	81	77	73	76

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TABLE 7

Retinol stability in the presence of Daidzein

Time (hours)	0% Daidzein	0.0001%	0.001%	0.01%
8	92	82	80	73
24	86	78	79	69
48	81	76	76	68

10 Retinol alone in the absence of any agents degraded slowly (8% by 8 hours, 14% by 24 hours and 19% by 48 hours). However, in the presence of genistein and daidzein the degradation of retinol was accelerated. As early as 8 hours, 16-18% of retinol was degraded in the presence of these flavonoids. This suggests that both genistein and daidzein caused marked increases in the instability of retinol. This will make it necessary to use special packaging, one  
15 compartment for retinol and another for the flavonoids in products containing retinoids and the flavonoids.

While the present invention has been described herein with some specificity, and with reference to certain preferred embodiments thereof, those of ordinary skill

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in the art will recognize numerous variations, modifications and substitutions of that which has been described which can be made, and which are within the scope and spirit of the invention. It is intended that all of these modifications and variations be within the scope of the present invention as described and claimed herein, and that  
5 the inventions be limited only by the scope of the claims which follow, and that such claims be interpreted as broadly as is reasonable. Throughout this application, various publications have been cited. The entireties of each of these publications are hereby incorporated by reference herein.

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